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Sex Determination in Dioecious *Silene latifolia*[1]

Effects of the Y Chromosome and the Parasitic Smut Fungus (*Ustilago violacea*) on Gene Expression during Flower Development

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We have embarked on a molecular cloning approach to the investigation of sex determination in *Silene latifolia* Poiret, a dioecious plant species with morphologically distinguishable sex chromosomes. One of our key objectives was to define a range of genes that are up-regulated in male plants in response to Y chromosome sex-determination genes. Here we present the characterization of eight male-specific cDNA sequences and classify these according to their expression dynamics to provide a range of molecular markers for dioecious male flower development. Genetically female *S. latifolia* plants undergo a partial sex reversal in response to infection by the parasitic smut fungus *Ustilago violacea*. This phenomenon has been exploited in these studies; male-specific cDNAs have been further categorized as inducible or noninducible in female plants by smut fungus infection. Analysis of the organ-specific expression of male-specific probes in male and female flowers has also identified a gene that is regulated in a sex-specific manner in nonreproductive floral tissues common to both male and female plants. This observation provides, to our knowledge, the first molecular marker for dominant effects of the Y chromosome in nonreproductive floral organs.

Dioecy is an outbreeding mechanism in which male and female reproductive organs develop on separate individuals. This breeding strategy is used by approximately 4% of angiosperm species (Yampolski and Yampolski, 1922). However, only five families of flowering plants are known to contain dioecious species with morphologically distinguishable sex chromosomes (Parker, 1990). *Silene latifolia* Poiret (white campion), also known as *Silene alba* (Miller) E.H.L. Krause and *Melandrium album* (Miller) Garcke, and its close relative, *Silene dioica* (L.) Clairv. (red campion), are examples of such dioecious species within the Caryophyllaceae family. *S. latifolia* and *S. dioica* are so closely related that crosses between them result in fertile hybrid progeny. Individual plants of these two species are either male ($2n$ = 22,XY), bearing staminate flowers, or female ($2n$ = 22,XX), bearing pistillate flowers. They exhibit strict genetic control of sex expression in which the Y chromosome exerts a dominant influence to promote male development (Westergaard, 1940). This type of chromosomal sex determination contrasts with those of other plant species such as *Rumex acetosa* (wood sorrel), in which a dosage-compensation mechanism based on the X chromosome-to-autosome ratio determines the sex of individual plants (Ainsworth et al., 1995). Various types of monoecious and dioecious plant-breeding strategies have been recently reviewed by Dellaporta and Caulderon-Urrea (1993) and Grant et al. (1994a).

The chromosomal genetics of *S. latifolia* sex determination have been investigated in mutant male plants that contained fragmented Y chromosomes (Westergaard, 1946, 1958; Ye et al., 1991; Grant et al., 1994a). The metacentric Y chromosome can be orientated by observation of its pairing with the X chromosome in meiosis. The sex chromosomes pair over pseudoautosomal regions, which extend approximately one-fourth of the length of the X chromosome from the telomere of its longer arm. Plants that contained deletions from the Y chromosome were found to show dramatic changes in sex expression. The loss of fragments of the Y chromosome distal to the pseudoautosomal region produced plants bearing hermaphroditic flowers. The loss of parts of the Y chromosome in its centromeric region, however, led to plants bearing sterile flowers that contained no third- or fourth-whorl sex organs. From these studies it was inferred that dominant genes at separate locations on the Y chromosome are separately responsible for the inhibition of pistil development and the promotion of stamen development in male plants. Westergaard (1958) demonstrated that another Y chromosome region is necessary for pollen fertility, and therefore postulated two effects of the Y chromosome on stamen development.

The early stages of floral organ primordium formation, as revealed by scanning electron microscopy, occur similarly in male and female plants of *S. latifolia* (Grant et al., 1994b). Sex-specific differences between male and female flowers arise subsequent to floral primordium formation by the selective arrest of the development of pistils in male flowers and of stamens in female flowers. The arrest of stamen development in female flowers occurs at a later stage than does the arrest of carpel development in male flowers, reinforcing the genetic evidence that two distinct

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Abbreviations: GRP(s), Gly-rich protein(s); ORF, open reading frame.

mechanisms operate to promote stamen development and arrest pistil development in male plants. A further difference in development between male and female flowers lies in the state of health of the arrested tissues; the arrested pistil in mature male flowers consists of a thin stalk of healthy, living tissue some 2 mm in length, whereas the rudimentary stamens produced in female flowers degrade during the later stages of floral development. Some additional sex-specific differences occur in *S. latifolia* other than those that affect the development of stamens and pistils. One difference is in sepal venation; the calyx tube in female flowers possesses 20 main veins in contrast to the 10 in male flowers. Additionally, female flowers are 50% larger than male flowers at maturity, and inflorescence morphology varies between the sexes (male inflorescences contain greater numbers of nodes and consequently produce more flowers than do female inflorescences).

The type of organs formed in each of the four floral whorls of angiosperms are attributed to the action of MADS-box homeotic genes (Weigel and Meyerowitz, 1994). Such homeotic genes have been intensively studied in the bisexual species *Antirrhinum majus*, and sequences closely related to these have now been reported in *S. latifolia* (Hardenack et al., 1994). From a comparison of the expression patterns of MADS-box genes in *S. latifolia* and *A. majus*, it would appear that floral organ identity is controlled similarly in these two species. It is apparent from these studies that the sex determination system in *S. latifolia* operates largely downstream from the processes that define floral organ identity. The critical sequences that control sex determination in *S. latifolia* remain to be identified, and these must act either following, or cooperatively with, MADS-box floral homeotic genes.

The sex-determination system of *S. latifolia* robustly resists perturbation by environmental conditions or exogenous plant hormone application (Ye et al., 1991; Grant et al., 1994a), in contrast to those of several other dioecious species such as *Mercurialis annua* (Louis, 1989), *Cannabis sativa* (Galoch, 1978), and *Arisaema triphyllum* (Policanski, 1981). In *M. annua*, for example, auxins and cytokinins act antagonistically to determine the sex of the plant, whereas in *A. triphyllum* nutrient availability modulates sex expression. However, following the systemic infection of *S. latifolia* or *S. dioica* by the parasitic smut fungus *Ustilago violacea (Mycobotryum violaceum)*, stamen development occurs in plants that are genetically female. Smut-infected anthers of *S. latifolia* are reported to contain little or no tapetal tissue, which would nourish the developing microspores and contribute to the pollen coating in healthy male plants (Audran and Batcho, 1981), causing pollen grains not to form. These anthers fill instead with the teliospores of *U. violacea*, thereby facilitating the propagation of the fungal parasite. Spores of *U. violacea* also accumulate in the developing ovary tissues of infected female plants (Batcho and Audran, 1981). The pistils of smut-infected females develop well beyond the stage of pistil arrest in males, although they do not reach full size and are infertile. One further modulation of sex expression, in this case releasing pistil development in male plants, has also been investigated. The treatment of genetically male *S. latifolia* plants with the nucleoside analog 5-azacytidine, an inhibitor of DNA methyltransferases, has been found by Janousek et al. (1996) to cause a proportion of flowers on genetically male plants to become hermaphroditic.

The emerging picture of *S. latifolia* sex determination is one of two main developmental processes, the arrest of pistil development and the promotion of stamen development, both of which occur in male plants. These two processes are controlled by genes mapping to two separate locations on the Y chromosome. In female plants, the absence of a Y chromosome results in uninterrupted pistil development and the absence of a cue for continued stamen development. The Y chromosome sex-determination genes act in male plants following the initiation of floral organ primordia and the partial development of third- and fourth-whorl floral organs. Y chromosome genes control the expression of other genes expressed during floral organ development. Therefore, we may postulate the operation of a control hierarchy, with Y chromosome genes involved at some point in the regulation of all other genes that are differentially regulated in male (compared with female) plants.

No requirement exists for the many sex-specifically expressed genes acting downstream of Y chromosome sex-determination genes to reside on the Y chromosome. Stamens are formed in smut-infected female plants, indicating that most of the genes required for male development must be located on the autosomes or the X chromosome. It has not been possible to demonstrate the involvement of any of the conventional plant growth regulators in *S. latifolia* sex determination. However, the capacity of *U. violacea* infection to stimulate stamen development suggests a possible role for some diffusible, small molecule acting as a signal in at least part of the sex-determination process. Key questions relating to such a *U. violacea*-derived signal include its precise chemical nature, its relationship to the corresponding Y chromosome-derived signal, and its point of action in the developmental hierarchy that controls stamen development.

MATERIALS AND METHODS

Seeds of *Silene latifolia* cv Poiret were obtained from John Chambers Seeds (Barton Seagrove, Northamptonshire, UK) and stocks were maintained through sexual propagation without close inbreeding. *S. latifolia* plants were grown in a greenhouse equipped with supplementary lighting to extend the photoperiod to 16 h. Flower tissue from *Silene dioica* (L.) Clairv. infected with the smut fungus *Ustilago violacea* was collected on dry ice from a natural population growing at Grass Wood (Grassington, North Yorkshire, UK).

Preparation of RNA and Construction of cDNA Libraries

Total RNA samples were prepared from various *S. latifolia* tissues (Scutt, 1997) and RNA concentrations were measured by UV spectrophotometry. For the construction of cDNA libraries, polyadenylated mRNA was purified from total RNA of male and female *S. latifolia* flower buds covering a developmental range from floral organ initia-

tion to immediately before anthesis. Polyadenylated RNA was selected by annealing to biotin-derivatized oligo[dT], followed by magnetic capture using streptavidin-coated paramagnetic particles (PolyAtract, Promega). Polyadenylated RNA preparations were copied to cDNA, from which directionally cloned male and female cDNA libraries were prepared using a bacteriophage λ-cDNA cloning kit (Unizap II, Stratagene) according to the manufacturer's instructions. Male and female flower bud primary cDNA libraries of 10^6 plaque-forming units were generated and amplified for further use in the preparation of a subtracted male cDNA library.

Subtraction of cDNA Libraries

A subtracted *S. latifolia* male flower cDNA library was prepared by a novel method termed "high-stringency subtraction," which is fully described elsewhere (Scutt and Gilmartin, 1997). The amplified male and female flower bud libraries constructed as described above were mass-excised in vivo to single-stranded recombinant pBluescript phagemid libraries by superinfection with VCS M13 helper phage. Phagemid DNA preparations of the male-excised library were subtracted using biotinylated PCR products that had been amplified from the female flower cDNA library. After two rounds of subtraction, the remaining recombinant phagemids were recovered by electrotransformation of *Escherichia coli* to create a subtracted cDNA library containing a high proportion of male flower-specifically expressed cDNAs.

Differential Screening of Subtracted cDNA Libraries

Duplicate sets of cDNA dot-blots were prepared from the subtracted male flower cDNA library and processed for differential screening using male and female cDNA radiolabeled probes (Scutt and Gilmartin, 1997). Autoradiographic signals from differential hybridizations were quantified and compared using a computer-controlled scanning densitometer. Those cDNAs showing male-enhanced expression were selected for further analysis. In certain cases cDNAs selected by differential screening of the subtracted library were used as probes to select longer or full-length homologous cDNAs from the male λ-cDNA library by plaque-lift hybridization (Sambrook et al., 1989).

RNA Gel-Blot Analysis

Total RNA samples, 10 μg per track, were fractionated by electrophoresis on 1% (w/v) agarose gels in the presence of formaldehyde (Sambrook et al., 1989) and blotted onto Hybond-N membranes (Amersham) according to the manufacturer's instructions. Parallel tracks containing RNA molecular size markers (Promega) were removed from RNA gel blots and stained in a 1% (w/v) solution of methylene blue.

RNA gel blots were hybridized with radiolabeled cDNA probes prepared by random prime labeling (Feinberg and Vogelstein, 1983) in solutions containing 50% (v/v) formamide (Sambrook et al., 1989). All blot hybridizations were finally washed to a stringency that permitted hybridization only between nearly perfectly matched duplexes. Final washing of blots was performed in solutions containing 0.1× SSC and 0.1% (w/v) SDS at 65°C (20× SSC is 3.0 M NaCl, 0.3 M trisodium citrate, pH 7.25). RNA gel blots were reprobed with a *S. latifolia* β-ATPase clone, a transcript that appears to be present at near uniform levels in most plant tissues, to demonstrate integrity of RNA samples and comparative loading of tracks.

Southern-Blot Hybridizations

Genomic DNA from *S. latifolia* male and female leaf tissues was prepared by the method of Graham (1978) and digested with suitable restriction endonucleases. Restriction digests were fractionated by electrophoresis on 0.7% agarose gels, 10 μg per track, in TAE buffer (Sambrook et al., 1989) before processing for Southern blotting onto Hybond-N membranes (Amersham) according to the manufacturer's protocols. Southern-blot hybridizations were carried out in aqueous solutions. Labeling of cDNA probes and washing conditions after Southern-blot hybridizations were identical to those used for northern-blot hybridizations.

DNA Sequence Determination and Analysis

Sequences of male-specific cDNAs were completely determined from both DNA strands. DNA samples were subjected to Taq polymerase thermal cycle sequencing reactions incorporating dye-derivatized, dideoxy chain terminators, and analyzed on an automated DNA sequencer (model 373A, Applied Biosystems). Similarity searches were carried out on predicted peptide sequences using the BLAST search method (Altschul et al., 1990) against the SwissProt database and on DNA sequences using the FASTA search method (Pearson and Lipman, 1988) against the EMBL and GenBank databases.

Scanning Electron Microscopy of *S. latifolia* Flower Buds

Specimens of male flower buds were prepared for scanning electron microscopy as described by Cohen (1979) and examined microscopically (CamScan Series 3, CamScan Analytical, Cambridge, UK).

RESULTS

Isolation of Male-Specific cDNA Sequences Expressed in Male *S. latifolia* Flowers

The starting point of the work presented here was the construction and differential screening of male and female flower cDNA libraries. As a part of the work, a modified cDNA subtraction protocol for the efficient isolation of differentially expressed cDNA sequences from λ libraries was developed. This protocol is outlined here; its full experimental details are presented by Scutt and Gilmartin (1997). After subtraction of a male flower cDNA library with a probe derived from female flower buds, a subtracted library of 180 cDNA clones was generated. Preliminary screening of this subtracted library with radiolabeled first-

strand cDNA probes identified a range of cDNA sequences that demonstrated clearly enhanced expression in male flowers compared with female flowers (data not shown). Comparison of a selection of cDNAs was made following partial sequence analysis; the longest member of each group of homologous clones was selected for further study. As necessary, longer or full-length cDNA sequences homologous to the partial cDNAs were selected from a male cDNA library by plaque-lift hybridizations. These cDNAs were designated with the acronym *Men* for Male *en*hanced. We present the detailed temporal and spatial expression dynamics of eight cDNAs, *Men-1* to *Men-8*, which are expressed in male, but not female, flower buds.

Men cDNA Sequences Encode Both Novel and Previously Characterized Classes of Proteins

Full nucleotide sequence data were determined from both strands of *Men-1* to *Men-8* that had been selected for further study from a subtracted cDNA library of *S. latifolia* male flower buds. Analysis of these data revealed that some of the *Men* cDNAs encoded predicted proteins that fell into known categories, whereas others encoded entirely novel classes of predicted proteins. Figure 1 shows peptide sequence data predicted from the eight cDNA sequences. A brief overview of the features of interest and homology to previously characterized sequences shown by cDNAs *Men-1* to *Men-8* and their predicted proteins is presented below and summarized in Table I, which also gives database accession numbers for the nucleotide sequences of the eight cDNAs.

The *Men-1* cDNA was unusual in that it contained no ORF of significant length compared with the length of the cDNA. The longest ORF in *Men-1* encoded a predicted peptide of only 36 amino acids (Fig. 1) from a possible ATG start codon at bases 70 to 72, which was preceded by an in-frame stop codon. The *Men-1* cDNA was similar in size to its mRNA as determined by RNA gel-blot analysis (Table I). Furthermore, of 20 independent homologs of *Men-1* selected from a male flower cDNA library, none were longer than *Men-1*, suggesting that this represents a nearly full-length transcript. However, additional mapping experiments will be required to determine whether this cDNA represents the major *Men-1* mRNA. No close similarity to previously characterized sequences could be found in the EMBL or GenBank databases by searching with either the *Men-1* cDNA or its short predicted polypeptide.

The proteins predicted from cDNAs *Men-2*, *Men-3*, and *Men-4* were GRPs containing 23, 36, and 28% Gly residues, respectively (Fig. 1). *Men-2* and *Men-3* were partial cDNAs (Table I), whereas *Men-4* was similar to the estimated size of its mRNA and contained an ATG start codon at bases 8 to 10 (Table I). Seven independent homologs of *Men-4*, selected by plaque hybridizations from a male flower cDNA library, shared the same 5′ end to within a few bases and no longer homologs were obtained, suggesting that *Men-4* is a full-length clone. The Gly residues in the Men-2- and Men-3-predicted polypeptides occurred in groups of up to four. In the case of Men-2, these Gly residues were found principally in the N-terminal half of the partial peptide sequence (Fig. 1). In addition to repetitive Gly clusters, the Men-2-predicted polypeptide also contained a sequence of 10 alternating Pro and Ala residues near its C terminus.

In the Men-4-predicted polypeptide, Gly residues occurred principally in two homopolymeric stretches of Gly-13 and also in repeating motifs of Gly-Gly-X and Gly-X-Gly, where X was most frequently Lys. Hydropathy analysis of the Men-4-predicted protein (data not shown) by the method of Eisenberg (1984) suggested the presence of a signal peptide, although the consensus signal cleavage motif of Ala-X-Ala (Briggs and Gierasch, 1986) was absent. The *Men-4* cDNA showed 95% nucleic acid identity with *MROS2*, a GRP encoding a cDNA of unknown function, which was also recently cloned from male flowers of *S. latifolia* (Matsunaga et al., 1996). The peptides encoded by

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Men-1:   1 MALSFATDTL KPTFLALPAF PPTQVASDGD NVSAVI

Men-2:   1 RHECNVAGGG GEGWGSGGSG GRGGQGWGSG GWGSGRSSDQ SPNLDKLFSS TTEEKQRQRQ RKDIKGNREQ
        71 NKEMIATGTT PAPAPAPAPA SG
                       Pro/Ala-rich region

Men-3:   1 HGCSDCGTGE GGGQGWGNDG SGSGGQQGWG SGWGDGWCSS GSGSGNGQGW GMGGGCQGRS CEGSGGYGEG
        71 EGRAWGTPGM NRAKIGHGKS AEKKQMVAMA PASE
                        ATP/GTP binding motif

Men-4:   1 MGSLNNTSLL PLSVLLILTS PIHIYSASRL LAEESSISEK VVNGQTKSIT SITGWFGGFP GGKDGKGGKG
        71 GKGGKGGGGG GGGGGGGGKE GGGGGGGGGG GGGANIPPRA AGEGLGIRSW GSGWGQGLDS SPGFDKLVPS
                poly-glycine regions
       141 ETEEKQLNLH QHTEAGKNQN DHETKPKELK GGQSNEEGEH AMAPTSE

Men-5:   1 MATKSCLLFI SLTVIIANAV SADRLHVVDR NRASEGIGVP QPDVNVVSAD RLHVVDRNRA SEGLGVHQTD
             signal cleavage motif       repeat 1                     repeat 2
        71 ANAVSADKLH VVDRNPASKG LGVHQTDANA VSADRPHMVD RNLASEGLGV SKDTNVNMMS KMGSAGKMNN
                      repeat 3                     repeat 4
       141 VVSPVVPVPM PGADGQFQFQ IGPIGFGWNW NFGGGVPGPL ITYSQPIVVS NGNGMNGMNA PYSTPSGLPF
       211 PYIGVMPSPG AYQPLPGYSG FYPGGYHRHP FGRYGPNPSG QHAQGANQMQ RPASGHD

Men-6:   1 SPESFSLSSP ESYTQTSPYV LNLNLPPHSP KYSHPHKHPH TTVFIIVIEC TIA
                                      Pro/His-rich region       prenylation motif

Men-7:   1 FVAFLSLLFS RQVLGLDKPM AGPACSDVIP KVTFCLLYIT GGSPSPSDAC CNGIKTVAST VKDKNDAVLV
                                      conserved Cysteine residues
        71 CNCLKDKLVD LQYQPSLIAS LSDKCSVSFK LPAISKATDC SKVNPPYFMM STNKAILKN

Men-8:   1 MANNMKSATF CKATWAIFLV ALAILVQLKG SEAQAGGCAS QLGNLNVCAP YVVPGAVNTN PSQECCAALS
                                 signal cleavage motif
        71 GVNHDCMCNT LRVASQLPSS CNLAALNCGN
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Figure 1. Polypeptide sequences predicted from *Men* cDNAs. Peptide motifs and other structural features described in the text are shown in boldface and are labeled below the sequences. Four direct repeats in the *Men-5*-predicted peptide are underlined and labeled below the sequence.

Table I. *A summary of structural features of cDNAs Men-1 to Men-8 and their predicted polypeptides*

Sequence Characteristics	cDNA							
	Men-1	*Men-2*	*Men-3*	*Men-4*	*Men-5*	*Men-6*	*Men-7*	*Men-8*
Length of mRNA (from RNA gel blots)	670 b[a]	1260 b	980 b	840 b	1050 b	1020 b	810 b	550 b
Length of cDNA (excluding poly[A] tail)	562 bp	439 bp	447 bp	732 bp	1001 bp	308 bp	569 bp	540 bp
Longest ORF and predicted protein	108 bp/36 Amino acids	276 bp/92 Amino acids	312 bp/104 Amino acids	567 bp/189 Amino acids	801 bp/267 Amino acids	109 bp/53 Amino acids	387 bp/129 Amino acids	300 bp/100 Amino acids
Abundance in male flower cDNA library	0.12%	0.21%	Not determined	0.29%	0.01%	Not determined	Not determined	Not determined
Homologies and structural features of interest for predicted protein	Very short predicted peptide	GRP	GRP; ATP-/GTP-binding motif	GRP homologous to MROS2; signal peptide	Four N-terminal repeats; signal peptide	Prenylation motif	Nonspecific lipid-transfer protein	*A9* tapetum-specific gene; signal peptide
EMBL database accession no.	Y08773	Y08774	Y08775	Y08776	Y08777	Y08778	Y08779	Y08780

[a] b, Bases.

these two cDNAs showed 86% identity (91% if conservative substitutions are allowed). Searches of the EMBL and GenBank databases with the *Men-2* and *Men-3* sequences failed to show close similarity to any particular subgroup of GRPs. Searches for peptide sequence motifs among the three Men-predicted GRPs revealed a consensus ATP/GTP-binding domain (Walker et al., 1982) in Men-3.

Men-5 showed no close similarity to previously characterized sequences in the EMBL or GenBank databases. The *Men-5* cDNA was similar in length to its mRNA and contained a putative ATG start codon at bases 13 to 15. The Men-5-predicted polypeptide of 276 amino acids (Fig. 1) was suggested by hydropathy data (not shown) to contain a putative N-terminal signal peptide associated with a signal cleavage motif. Four inexact direct repeats, each one 27 amino acids in length, were also present in the Men-5-predicted peptide sequence immediately following the putative signal peptide. Each of these four repeats contained a hydrophilic region followed by a region of sufficient length and hydrophobicity (data not shown) to be membrane-spanning.

The *Men-6* cDNA showed no similarity to previously identified sequences in the EMBL or GenBank databases. *Men-6* was a partial cDNA, encoding 53 amino acids at the C terminus of a predicted polypeptide (Fig. 1). The Men-6-predicted polypeptide contained a putative prenylation site (Fig. 1; Table I), suggesting the possibility of its attachment to a membrane via farnesyl or geranylgeranyl groups (Glomset et al., 1990). The predicted Men-6 peptide sequence also contained a short region rich in His and Pro (Fig. 1), which resembled homopolymeric regions of unknown function found in some homeodomain and other regulatory proteins from *Drosophila melanogaster*, such as that encoded by the ecdysone-inducible *E74* gene (Burtis et al., 1990).

Men-7 and *Men-8* were both identified as homologous to previously characterized genes. *Men-7* encoded the C-terminal 129 amino acids of a predicted polypeptide (Fig. 1) with homology to nonspecific lipid-transfer proteins. The Men-7-predicted protein included eight Cys residues, indicated in Figure 1, which are conserved between most known nonspecific lipid-transfer proteins and are believed to form intramolecular disulfide bridges. No ATG start codon was apparent in *Men-7*. However, the Men-7-predicted protein was of approximately equal length to other nonspecific lipid-transfer proteins, which suggested that its cDNA is close to full-length. A highest amino acid sequence similarity of 34% (56% if conservative substitutions are allowed) (data not shown) to the Men-7-predicted polypeptide was shown by a nonspecific lipid-transfer protein from the endosperm of germinated *Ricinus communis* seeds (Takishima et al., 1986).

Men-8 was found to be homologous to the *A9* tapetum-specific genes (Paul et al., 1992) from *Brassica napus* (42% amino acid similarity, or 63% if conservative substitutions are allowed) and *Arabidopsis thaliana* (43% amino acid similarity, or 56% if conservative substitutions are allowed) (data not shown). Compared with these sequences the *Men-8* mRNA appeared to be full-length, with an ATG start codon at bases 10 to 12. Hydropathy data (not shown) indicated that the Men-8-predicted polypeptide includes a putative N-terminal signal peptide sequence associated with a signal cleavage motif matching the Ala-*X*-Ala consensus (Fig. 1).

A Subgroup of *Men* cDNAs Is Up-Regulated in Smut-Infected Female Flowers

Following the differential screening of a subtracted male cDNA library from *S. latifolia* flower buds and the subse-

quent sequencing of cDNA clones, attention was focused on eight cDNAs that showed differential signals from male over female flower bud cDNA probes. To confirm these eight cDNAs to be expressed specifically in male flower buds, they were used as hybridization probes against RNA gel blots of total RNA from male and female flower buds of *S. latifolia* harvested over a broad range of development. On the same RNA gel blots, samples were included from genetically female *S. dioica* plants that had been systemically infected with *U. violacea*. The flowers of these plants contained stamens in addition to partially developed pistils.

The results of RNA gel-blot analyses using eight different *Men* cDNAs as hybridization probes against male, female, and smut-infected female flower bud RNAs are presented in Figure 2. Two distinct patterns of expression were distinguishable: *Men-2* (Fig. 2B), *Men-3* (Fig. 2C), *Men-5* (Fig. 2E), *Men-7* (Fig. 2G), and *Men-8* (Fig. 2H) showed hybridization to male flower RNA, whereas *Men-1* (Fig. 2A), *Men-4* (Fig. 2D), and *Men-6* (Fig. 2F) showed hybridization to RNA from both male flower buds and smut-infected female flower buds. None of the *Men* cDNAs showed expression in uninfected female flower buds. RNA gel-blot filters were reprobed with a nominally constitutive *S. latifolia* β-ATPase clone (M. Shenton and P.M. Gilmartin, unpublished data) as a control for RNA loading and integrity (Fig. 2I). The data are presented as panels for ease of comparison. The relative positions of bands do not reflect transcript sizes; these are presented along with other comparative data in Table I.

This study demonstrates a molecular distinction within the eight *Men* cDNAs presented. Five of these *Men* cDNAs are expressed only in male plants in response to developmental cues derived from the Y chromosome, whereas the remaining three cDNAs are also expressed in female plants in response to infection by *U. violacea*. The five cDNAs *Men-2*, *Men-3*, *Men-5*, *Men-7*, and *Men-8*, which were induced solely in male flower buds, must either be encoded by genes located on the Y chromosome or be expressed in

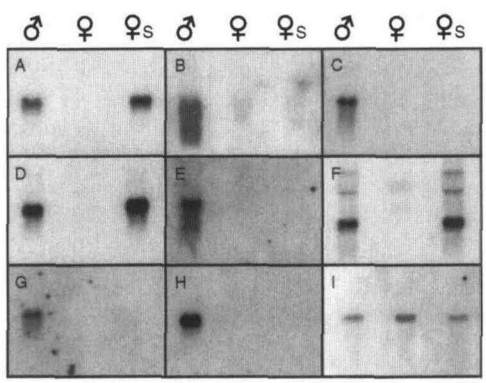

Figure 2. RNA gel-blot analyses of male, female, and smut-infected female *S. latifolia* flower RNA with *Men* cDNA probes. Gel blots of total RNA from *S. latifolia* flower buds were hybridized to radiolabeled cDNA probes of *Men-1* (A), *Men-2* (B), *Men-3* (C), *Men-4* (D), *Men-5* (E), *Men-6* (F), *Men-7* (G), and *Men-8* (H). A *S. latifolia* β-ATPase probe was used to demonstrate RNA integrity and relative loading levels (I).

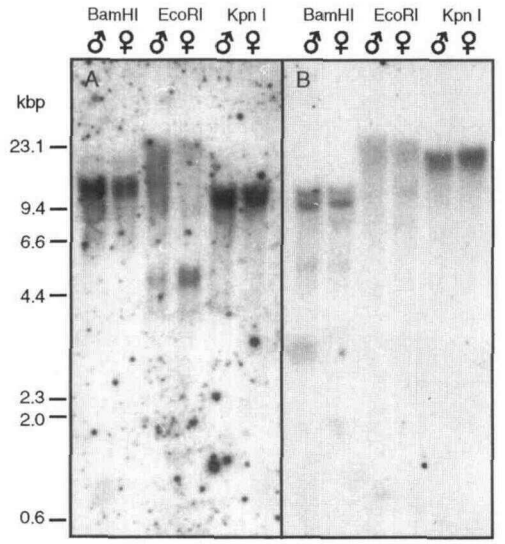

Figure 3. Genomic Southern-blot analysis of *S. latifolia* with *Men* cDNA probes. Southern blots of genomic DNA from male and female plants, cleaved with restriction endonucleases as shown, were hybridized to probes of *Men-3* (A) and *Men-5* (B).

response, directly or indirectly, to Y chromosome genes that control sex determination. The other three *Men* cDNAs, *Men-1*, *Men-4*, and *Men-6*, were expressed both in male plants and in smut-infected female plants. Genes encoding these, common to both sexes, must be located on the autosomes or X chromosome and can therefore be excluded as Y chromosome sex-determination genes.

The Genes *Men-1* to *Men-8* Are Present in Both Male and Female Plants

Probes homologous to genes located on the larger part of the Y chromosome of *S. latifolia*, which does not recombine with the X chromosome at meiosis, would be expected to reveal male-specific banding patterns on Southern-blot hybridizations to male and female *S. latifolia* genomic DNAs. After the identification of five male-specific cDNAs that were not induced in female plants in response to smut fungus infection, the genomic organization of these sequences was investigated to determine if any of them were unique to male plants. Genomic Southern blots of restriction endonuclease-digested genomic DNA from male and female plants were probed individually with the *Men* cDNAs. Two representative genomic Southern blots are shown in Figure 3, A and B, probed with *Men-3* and *Men-5*, respectively. Southern-blot analyses using probes derived from the five *Men* cDNAs that were not up-regulated in smut-infected female plants, *Men-2*, *Men-3*, *Men-5*, *Men-7*, and *Men-8*, showed similar banding patterns from male and female genomic DNAs. Slight discrepancies in Southern-blot banding patterns between male and female plants, some of which are apparent in Figure 3, can be explained by allelic differences within the polymorphic population of plants from which DNA was prepared, or by partial digestion of genomic DNA by restriction enzymes in certain cases. This phenomenon was routinely encoun-

tered in Southern blots of *S. latifolia* DNA, including those probed with genes equally expressed in both male and female plants (data not shown).

The results derived from Southern-blot analyses such as those presented in Figure 3, coupled with the demonstration that three *Men* cDNAs are expressed in smut-infected female plants (Figs. 2, A, D, and F), indicate that all eight genes encoding the *Men* cDNA sequences characterized here are present in both male and female plants. None of the eight *Men* cDNAs appear to be transcribed from Y chromosome genes unique to male plants, but all appear to be expressed in male plants in response to signals derived, directly or indirectly, from the Y chromosome.

The Eight cDNAs *Men-1* to *Men-8* Show Differing Temporal Expression Profiles

To investigate the temporal expression dynamics of the eight *Men* cDNA clones presented here through bud development, male flower buds ranging between 1 and 15 mm in length were grouped into five developmental stages for RNA preparation and RNA gel-blot analysis. Tissue covering these developmental stages and some younger material had previously been included in RNA preparations used for the construction of the subtracted cDNA library from which *Men-1* to *Men-8* were cloned. Scanning electron micrographs representing these five developmental stages are shown in Figure 4. Stage 1 contained material from immediately before meiosis until pollen wall formation following microsporogenesis. In buds at stage 1, the loculi of anthers were lined with the tapetum. Tapetal degradation occurred largely over stages 1 and 2. In buds at stage 2, petals were approximately 2 mm in length and tightly wrapped around the stamens. Over stages 3 and 4, petals were light green in color and elongated to approximately 4 and 8 mm, respectively. By stage 5, filament elongation was clearly evident and bud breakage occurred to reveal the tips of the petals. Also at stage 5, the petals became white in color after the accumulation of flavonoid pigments. In subsequent stages of *S. latifolia* male flower development, which are not illustrated and which did not feature in the temporal analysis of *Men* gene expression presented here, petal and filament elongation continued until flowers reached a length of approximately 25 mm, anthers dehisced to release mature pollen, and flowers opened fully.

The results of RNA gel-blot analyses using RNA derived from the five stages of male flower development presented in Figure 4, probed with eight male-specific *Men* cDNA sequences, are presented in Figure 5. These results demonstrate that the eight cDNA sequences fall into two main temporal expression classes, one early-expressed class and another expressed at all examined stages of male flower development. As can be seen from Figure 5, *Men-2* (Fig. 5B), *Men-3* (Fig. 5C), *Men-5* (Fig. 5E), *Men-7* (Fig. 5G), and *Men-8* (Fig. 5H) were expressed principally in flower buds at stage 1 (Fig. 4), whereas *Men-1* (Fig. 5A), *Men-4* (Fig. 5D), and *Men-6* (Fig. 5F) were expressed to a similar level throughout bud stages 1 to 5. The five *Men* cDNAs that showed expression predominantly in the earlier bud stages in Figure 5 are also those that were not induced in female plants infected with *U. violacea* (Fig. 2). Two of the three *Men* cDNAs encoding GRPs, *Men-2* and *Men-3*, are early-expressed and are not induced by smut infection, whereas the third, *Men-4*, is expressed throughout male bud development and is induced in smut-infected females. Therefore, the two major classes of expression dynamics exhibited by the *Men* cDNAs investigated cross any broad categories formed by consideration of sequence data.

The Eight cDNAs *Men-1* to *Men-8* Show Distinct Organ-Specific Expression Patterns

It was anticipated that male-specific expression of *Men* cDNAs would be due principally to expression in stamens, since these are the only organs unique to male flowers. However, the differences in sepal venation and inflorescence morphology between male and female plants suggested the possibility that the Y chromosome could also influence patterns of gene expression other than in the third and fourth whorls of the flower. To investigate the organ-specific expression profiles of the eight cDNAs, probes derived from *Men-1* to *Men-8* were hybridized in RNA gel-blot assays to RNA prepared from dissected floral organs and vegetative tissues of male *S. latifolia* plants. Floral organs for these studies were harvested over a range of developmental stages, predominantly from flower bud stages 3 to 5 (Fig. 4).

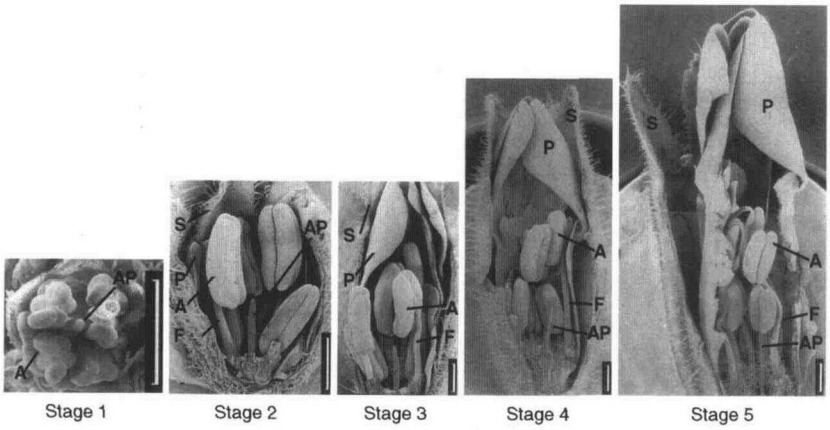

Figure 4. Scanning electron micrographs of *S. latifolia* male flower bud development. Male flower buds are shown partially dissected to reveal internal structures at successive stages of development. Stage 1 is shown in the transverse view; the remaining stages are shown in the longitudinal view. Arrested pistils (AP), anthers (A), sepals (S), petals (P), and filaments (F) are indicated on the micrographs; scale bars = 1000 μm.

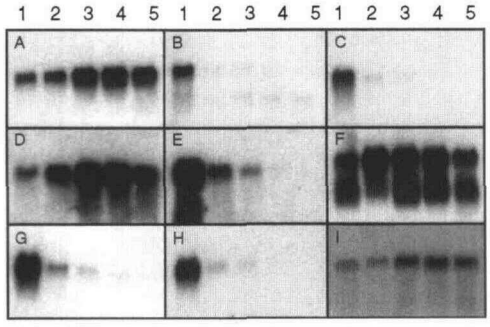

Figure 5. RNA gel-blot analyses of the temporal expression dynamics of *Men* cDNAs in *S. latifolia* male flower buds. Gel blots of total RNA from male flower buds at developmental stages 1 to 5 (as shown in Fig. 4) were hybridized to radiolabeled cDNA probes of *Men-1* (A), *Men-2* (B), *Men-3* (C), *Men-4* (D), *Men-5* (E), *Men-6* (F), *Men-7* (G), and *Men-8* (H). A *S. latifolia* β-ATPase probe was used to demonstrate RNA integrity and relative loading levels (I).

Because of their small sizes, routine dissection of floral organs from stages 1 and 2 (Fig. 4) was not possible.

The results of the analyses of organ-specificity of *Men* cDNAs are presented in Figure 6. All of the cDNA sequences analyzed were flower-specific; no expression was observed in vegetative tissues (Fig. 6, A–H). Furthermore, all of the probes gave their strongest hybridization signals with anther RNA (Fig. 6, A–H). Probes *Men-1*, *Men-4*, and *Men-6* (Fig. 6, A, D, and F) gave higher signals in anther tissue than the remaining five probes, which is consistent with the comparatively higher expression levels shown by these three cDNAs in the mature flower buds used in this analysis (Fig. 6, A, D, and F).

In addition to anther expression, clones *Men-4* and *Men-6* (Fig. 6, D and F) showed a comparatively low level of expression in filaments, although this may stem from gene expression in the region of attachment between anther and filament. The *Men-6* cDNA was the only clone investigated that showed expression outside the tissues of the stamen; in addition to a high level of anther expression, it also hybridized to male petal RNA (Fig. 6F). The observed hybridization signal of *Men-6* from petals is clearly much lower than that obtained from anthers (Fig. 6F), and the *Men-6* hybridization signal in male flower buds (Fig. 2F) was therefore principally derived from *Men-6* expression in anthers rather than petals.

All RNA gel blots were controlled by reprobing of a nominally constitutive β-ATPase probe (Fig. 6I). Figure 6D contains a composite RNA gel blot; however, both autoradiographs from which this figure was prepared showed whole male flower bud RNA tracks giving signal intensities equal to those of positive hybridization controls, so that signals from different tracks in Figure 6D correctly reflect the relative abundances of the *Men-4* transcript in total RNA extracts.

Men-6 Is a Male Flower-Specific cDNA Expressed in Both the Second and Third Floral Whorls

The investigation of organ-specific expression of *Men* cDNAs demonstrated that *Men-6* was expressed in anthers and, to a lower level, in petals of male *S. latifolia* flowers (Fig. 6F). The characterization of *Men-6* following cDNA library subtraction (data not shown) and by RNA gel-blot analysis to whole male and female flower bud RNAs (Fig. 2F) suggested that the expression of this clone was male-specific. However, the possibility existed that a low level of expression contributed from female petals in whole female bud RNA samples could have remained undetected in these experiments. To determine whether the observed expression of *Men-6* in petals was specific to male flowers, or if female petals also exhibited low levels of *Men-6* expression, the *Men-6* probe was used in gel-blot analysis of RNA isolated from sepals and petals of male and female *S. latifolia* flower buds. In addition, RNA from whole male flower buds was used as a control. These data are presented in Figure 7A. Figure 7B shows a control hybridization with a *S. latifolia* β-ATPase clone.

The *Men-6* probe revealed an abundant transcript in whole male flower buds, as would be expected from the high level of anther expression of this clone, and a less-abundant transcript of the same size in male petals (Fig. 7A). The petal expression observed from *Men-6* did not result from contamination of petal RNA samples with small quantities of anther RNA. Clones *Men-1* and *Men-4*, both of which showed high levels of anther expression (Fig.

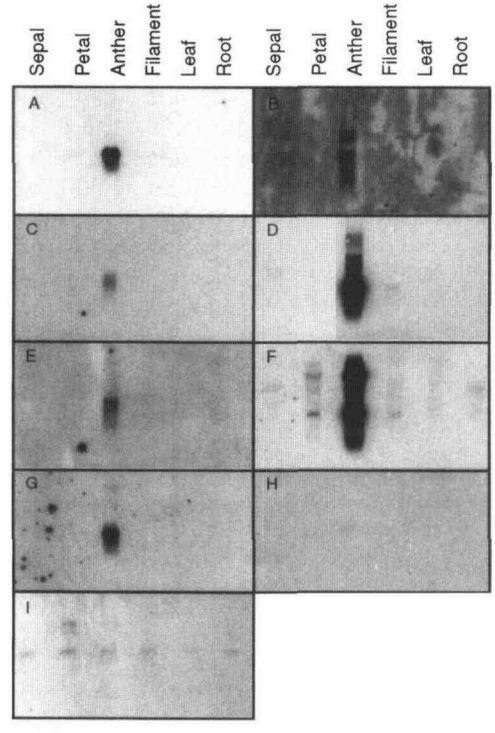

Figure 6. RNA gel-blot analyses of the organ-specific expression dynamics of *Men* cDNAs in male *S. latifolia* plants. Gel blots of total RNA from the dissected organs of male plants were hybridized to radiolabeled cDNA probes of *Men-1* (A), *Men-2* (B), *Men-3* (C), *Men-4* (D), *Men-5* (E), *Men-6* (F), *Men-7* (G), and *Men-8* (H). A *S. latifolia* β-ATPase probe was used to demonstrate RNA integrity and relative loading levels (I). D represents a composite RNA gel blot, of which both original autoradiographs showed whole male flower bud signals of equal intensity with the *Men-6* probe.

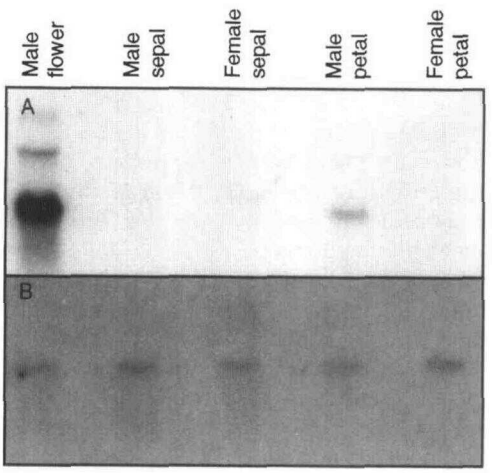

Figure 7. RNA gel-blot analysis of the expression of *Men-6* in sepals and petals of male and female *S. latifolia* flowers. A, Gel blot of RNA from whole male flowers and dissected floral organs probed with the *Men-6* cDNA. B, The same RNA gel blot is also probed with a *S. latifolia* β-ATPase clone to demonstrate RNA integrity and relative loading levels.

6, A and D), did not hybridize to male petal RNA in gel blots identical to that probed in Figure 7A (data not presented). No expression of *Men-6* was detected in the sepals from either male or female flowers or in the petals of female flowers. Therefore, this clone represents the first example, to our knowledge, of a plant sequence expressed differentially in a nonreproductive tissue as a consequence of sex determination.

DISCUSSION

Men cDNAs Provide Developmental Markers for the Molecular Analysis of Sex Determination in *S. latifolia*

The analysis of chromosomal deletion mutants previously performed in *S. latifolia* (Westergaard, 1946; Ye et al., 1991; Grant et al., 1994a) demonstrated that a male program of floral development is under the control of genes that reside on distinct regions of the Y chromosome. We have cloned eight cDNAs, *Men-1* to *Men-8*, the expression of which is ultimately induced in response to Y chromosome genes in male plants. Differences shown between the *Men* clones in timing and organ-specificity of expression, coupled with differences in the induction of expression in female flower buds after infection with *U. violacea*, have been used to group these cDNAs into distinct expression categories. From the differing expression dynamics of the *Men* genes presented, it may be concluded that they are up-regulated in male plants by a range of mechanisms, which must involve Y chromosome sex-determination genes operating at some level in a control hierarchy in each case. The various classes of *Men* cDNAs that have been characterized provide molecular markers for developmental processes occurring during male flower development. Such probes may be used to define separate elements of the sex-determination process in a range of situations, such as healthy male plants, female plants infected with *U. violacea*, and hermaphroditic deletion-mutant plants, which will form the basis of future studies.

Three *Men* cDNAs Are Expressed during Both Y Chromosome- and *U. violacea*-Induced Stamen Development

Three of the eight *Men* cDNAs investigated in this work, *Men-1*, *Men-4*, and *Men-6*, were expressed to approximately equal levels in the stamens of male plants and in those of female plants infected with *U. violacea*. It appears that these sequences are equally up-regulated in response to developmental cues supplied by Y chromosome sex-determination genes and by *U. violacea*. Because the anthers produced by smut-infected female plants contain little or no tapetum or sporogenous tissue, the three cDNAs, *Men-1*, *Men-4*, and *Men-6*, are most likely to be expressed in the only remaining anther tissue, the anther wall.

Although sequence analysis fails to provide proof of function of *Men-1*, *Men-4*, and *Men-6* in male flowers, a number of their structural features are worthy of comment. The most striking feature of the *Men-1* cDNA is its very short predicted peptide. The longest ORF predicted from this cDNA of 562 bp is only 36 amino acid residues in length. Isolation and characterization of 20 independent cDNA sequences suggests that *Men-1* is full-length, and the sequence analysis of four independent clones confirms the absence of any possible translation product longer than 36 amino acid residues. Alternatively, all 20 cDNAs could represent a reverse-transcription artifact in which the mature transcript is not efficiently reverse-transcribed because of a secondary structure, whereas a misspliced transcript is efficiently reverse-transcribed. RNase protection assays will distinguish between these possibilities.

It is not known whether the *Men-1* polyadenylated mRNA is translated in vivo to produce a polypeptide product. However, a precedent for such an mRNA is known from plants: the early nodulin gene, *ENOD40*, contains a very short ORF that is translated in vivo (van de Sande et al., 1996). The ENOD40 peptide of approximately 10 amino acids has been shown to act as a signaling molecule with a constitutive expression that leads to the uncontrolled production of adventitious roots. In the light of this recent discovery of the role of *ENOD40*, the roles of other genes containing very short ORFs, such as *Men-1*, may also become of interest. The possibility that the *Men-1* polyadenylated mRNA remains untranslated in vivo, however, cannot be excluded. Examples of such untranslated mRNAs, some of which fulfill regulatory roles, are known from animal systems (Rastinejad and Blau, 1993; Leighton et al., 1995).

Men-4 is predicted to encode a GRP homologous to another GRP-encoding cDNA, *MROS2*, which was recently cloned from *S. latifolia* (Matsunaga et al., 1996). The Men-4- and MROS2-predicted peptides share 86% sequence identity, which indicates that they are encoded by either two members of a gene family, or by two diverged alleles of a single gene. The *MROS2* cDNA is stamen-specific, as is *Men-4*, and its expression has been localized to specific regions of the anther wall, although its function is unknown. Both the Men-4- and MROS2-predicted proteins include cen-

tral polyglycine motifs, although Men-4 contains two of these and MROS2 contains only one. Further structural features of MROS2, highlighted by Matsunaga et al. (1996) as being of potential functional significance, include a repeating trimeric motif of GGKDGK and a motif of SPPPP, which flank the MROS2 polyglycine tract. These motifs, however, are not well conserved in the Men-4 predicted protein.

In addition to these *S. latifolia* sequences, other anther-specific GRPs are known from tobacco (*Nicotiana tabacum*) (Koltunow et al., 1990). The *Men-4* GRP is only moderately rich in Gly compared with other classes of GRPs, which contain up to 70% Gly (Condit and Meagher, 1986). Of the many classes of plant GRPs known, some have been ascribed roles, although the Men-4 and MROS2 predicted proteins do not resemble any class of GRPs with a known function. Some plant GRPs are secreted to the cell wall, where they fulfill structural roles, whereas others remain in the cytoplasm (Showalter, 1993). The predicted presence of a signal peptide suggests that the Men-4 GRP could potentially enter the secretory pathway to the cell wall.

The *Men-6* cDNA is a novel sequence that encodes a protein of unknown function. One potentially important structural feature shown by the Men-6-predicted protein is a putative prenylation site, indicating a potential C-terminal site of membrane attachment. However, the most interesting property of the *Men-6* cDNA is its expression pattern. The *Men-6* cDNA is expressed male-specifically in petals in addition to its high level of anther expression.

Five *Men* cDNAs Are Not Up-Regulated by *U. violacea* Infection

RNA gel-blot analyses were used to test for the induction of *Men* gene expression in female plants by smut infection, with the finding that five of the cDNAs investigated, *Men-2*, *Men-3*, *Men-5*, *Men-7*, and *Men-8*, were not up-regulated in this situation. It is clear that *U. violacea* is capable of supplying a signal early during female flower development, which leads to the formation of anthers. However, as is demonstrated here, only some of the male-specific genes characteristic of anther development in male plants are expressed in these organs. This may be explained by the structural differences between the anthers of male flowers and the smut-infected anthers of female flowers. The anthers of male flowers contain a tapetum and male germ-line cells, which eventually form mature pollen grains. In smut-infected female anthers, however, little or no tapetum or pollen is formed. It is not clear whether the smut fungus is unable to supply a developmental cue for tapetum and pollen formation, or whether its own growth and development inside the anther loculus disrupts the formation of these tissues. In either case, the lack of expression of a subgroup of *Men* cDNAs in smut-infected anthers, coupled with the observation that the same cDNAs are expressed only during early developmental stages in which the tapetum remains intact, strongly suggest that these cDNAs are expressed in the tapetum or male germ-line cells. This is reinforced by the identities of two of these cDNAs, *Men-7* and *Men-8*, which have homologs known to be pollen- or tapetum-specific in other plant species.

Sequence analysis of the five *Men* cDNAs that are not up-regulated by smut infection shows that three of them, *Men-2*, *Men-3*, and *Men-5*, represent novel classes of genes, and that the remaining two, *Men-7* and *Men-8*, are homologous to known classes of genes. *Men-2* and *Men-3* both encode novel GRPs, and the Men-3-predicted GRP contains a nucleotide-binding-site motif. Hydropathy analysis of the Men-5-predicted protein indicates four potentially membrane-spanning domains at its N terminus, suggesting that the N-terminal part of this protein may be inserted into a membrane in vivo. Men-7 encodes a protein with homology to lipid-transfer proteins, of which an anther-specific example from *N. tabacum* (Koltunow et al., 1990) and a pollen-specific example from *Parietaria judaica* (Costa et al., 1994) are known. Nonspecific lipid-transfer proteins, such as that predicted from *Men-7*, are able to transfer many categories of lipids and phospholipids across membranes and are possibly necessary for membrane biogenesis (Wirtz, 1991). The *Men-8* cDNA is homologous to the *A9* genes of unknown function from *B. napus* (Scott et al., 1991) and *A. thaliana* (Paul et al., 1992). These genes are known to be expressed specifically in the tapetum.

The Y Chromosome Modulates Gene Expression and Development in All Four Floral Whorls

The Y chromosome exerts major effects to promote stamen development and arrest carpel development in whorls three and four of male *S. latifolia* flowers. Additionally, the Y chromosome affects the development of the nonreproductive sepal organs of whorl one, which are fused into a calyx tube containing 20 main veins in females and 10 main veins in males. The effect of the Y chromosome on sepals may be secondary to its effect on other floral organs, although it is arguable that the Y chromosome is ultimately responsible for all of the differences exhibited between male and female plants of *S. latifolia*.

Although no gross morphological differences between male and female petals have been noted, the present work reveals a molecular distinction between male and female petals, which also can be ascribed to the action of Y chromosome genes. The *Men-6* cDNA has been shown to be up-regulated in the anthers and petals of male plants, but not in the petals of female plants. This observation extends the known sphere of influence of the Y chromosome in *S. latifolia* to all four floral whorls. In many cases, the signal transduction pathways linking Y chromosome genes to male-specifically expressed *Men* genes may involve numerous control steps. It is possible, however, that the transcription of some male-specific *Men* genes may be under particularly close control by sex-determination genes; good candidates for these are genes that are male-specifically expressed in tissues present in both male and female plants. Further analysis will determine whether *Men-6* expression in petals is closely or directly dependent on the presence of Y chromosome sex-determination genes.

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